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BRCA2 is a tumor suppressor gene which has been implicated in a large number of inherited breast cancers. We have generated a full-length human BRCA2 cDNA clone which incorporates silent mutations creating unique restriction sites. Furthermore, we describe a rabbit polyclonal antiserum raised against murine Brca2 which is suitable for both Western blotting and immunoprecipitation. We demonstrate that Brca2 is a nuclear phosphoprotein in a murine mammary epithelial cell line (NMuMG) , and we demonstrate that protein levels are regulated in multiple mouse cell lines during cell cycle progression. Additionally, we describe progress and plans in creating cell lines which inducibly alter Brca2 levels, and we anticipate using these cell lines to investigate the role of Brca2 in the cellular response to DNA damage and in cell cycle progression. Finally, we describe additional training opportunities which form an integral part of the nature of this grant.

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FOREWORD

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Introduction

As documented in the 1996-97 annual report for this grant, we have altered the strategy which we initially undertook (as described in the original grant proposal) to study the relationship between puberty and breast cancer risk. Consistent with the broader aims of that grant, we shifted the focus of our investigations to BRCA2, the second familial breast cancer gene. As we argued in last year's report, the fact that BRCA2 is upregulated in the murine mammary gland during puberty (Rajan *et al*, 1996) demonstrates that it is in fact the type of gene which we might have expected to identify using our initial approach. This report incorporates our results through September, 1998 in the context of an altered statement of work.

BRCA2 was initially identified as a tumor suppressor gene responsible for inherited breast cancer by classical linkage analysis (Wooster *et al*, 1994), and the gene was subsequently cloned and sequenced in 1995 (Wooster *et al*, 1995; Tavtigian *et al*, 1996). BRCA2 encodes a large protein (3418 AA) with no significant homology to any previously identified genes or structural motifs. The very large exon 11 of BRCA2 contains an array of 8 "BRC" repeats (Bork *et al*, 1996; Bignell *et al*, 1997); however, only one additional gene (encoding a *C. elegans* protein of unknown function) found in GenBank contained this novel consensus repeat. Thus, examination of the primary sequence of BRCA2 was unhelpful in elucidating hints as to its function.

One early clue to the function of BRCA2, however, has emerged from the work of a number of groups who have demonstrated that BRCA2 interacts with Rad51, a known DNA repair gene (Mizuta, *et al*, 1997; Wong *et al*, 1997; Katagiri *et al*, 1998; Chen *et al*, 1998). This suggests at least one way in which BRCA2 may prevent tumorigenesis: wild-type BRCA2 may be an integral part of the DNA repair machinery and thus may reduce the rate of accumulation of deleterious mutations. While initial knockouts of BRCA2 showed early embryonic lethality (Sharan *et al*, 1997; Suzuki *et al*, 1997; Ludwig *et al*, 1997), one group (Sharan *et al*, 1997) also reported that early-stage knockout blastocysts showed in increased sensitivity to ionizing radiation, thus lending further weight to this model. Furthermore, several subsequent knockouts which appear to retain partial BRCA2 function (in that they result in viable pups—hereafter these "knockouts" will be designated as "hypomorphs") have nonetheless reported hypersensitivity to gamma- (Connor *et al*, 1997; Morimatsu *et al*, 1998) or UV-irradiation (Patel *et al*, 1998). Inasmuch as BRCA1 has also been shown to interact with Rad51 (Scully *et al*, 1997a), has been implicated in the repair of DNA damage (Gowen *et al*, 1998), and colocalizes with BRCA2 during development (Rajan *et al*, 1997), it has been intriguing to speculate that these two proteins function in overlapping pathways (for the most recent development along these lines, see Chen *et al*, 1998).

While evidence has accumulated for a role of BRCA2 in DNA damage repair, this does not rule out a direct role for BRCA2 (particularly the N terminus) in the control of proliferation. *Brca2* is expressed in proliferating compartments of both the fetal and adult mouse (Rajan *et al*, 1997); further, the early embryonic lethality of *Brca2* knockout mice is due to defects in proliferation (this effect may be indirect, however—see Ludwig *et al*, 1997), and even the aforementioned "hypomorphs" show defects in growth.

Additionally, *Brca2* levels are regulated throughout the cell cycle (Rajan *et al*, 1996; see also Bertwistle *et al*, 1997); this is particularly intriguing in light of the recent observation that >60% of cell-cycle-regulated genes in the yeast *S. cerevisiae* have identified roles in cell cycle progression (Cho *et al*, 1998).

In light of such data, we have focused our efforts on examining the role of BRCA2 in the repair of DNA damage and in cell-cycle control.

Body

Specific Aim I. Determine the role of BRCA2 in the response to DNA damage

Task 1: Generate a full-length clone of BRCA2 (months 13-18)

Long-range PCR was used to amplify overlapping pieces of human *BRCA2* cDNA, and the resulting clone was confirmed by sequencing and comparison to published BRCA2 sequences. Mutations resulting from this PCR cloning strategy were corrected (MORPH, 5'-3'), and the relevant fragments were resequenced to ensure that no further mutations were introduced during this correction process. A single new mutation was corrected, and the resulting wild-type clone was assembled (Figure 1). In addition, mutations were introduced in order to create clones containing either the 999del5 (common in the Icelandic population—Thorlacius *et al*, 1996) or 6174delT (common in the Ashkenazi Jewish population—Oddoux *et al*, 1996) mutations. Thus, full-length wild-type and mutant BRCA2 clones were successfully constructed.

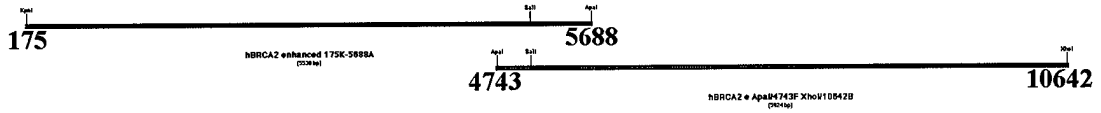

Figure 1: Construction of full-length human BRCA2 clone

After long-range PCR had been performed to generate two overlapping fragments of human *BRCA2* cDNA, the clones were sequenced. Mutations (defined as nonsilent deviations from the published BRCA2 sequences or common polymorphisms) were corrected and the clones were resequenced to confirm that no further mutations had been introduced. In addition, clones containing common mutations (999del5, 6174delT) were generated.

In order to facilitate further manipulation of BRCA2 despite the lack of single-cutting enzymes in its (large) coding sequence, we designed primers which would introduce silent mutations spaced across BRCA2 and which create unique restriction sites. This results in the construct which we have designated B2R (Figure 2).

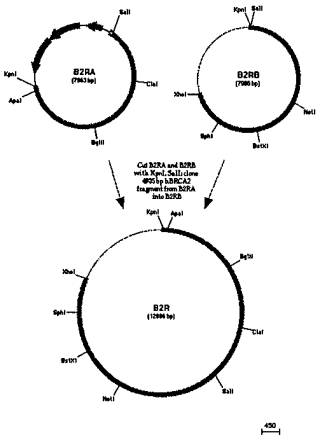

Figure 2: Construction of B2R

Primers were designed which incorporate silent mutations into *BRCA2* and which create unique restriction sites in order to allow manipulation of *BRCA2* sequence. This full-length construct (in pSP73 backbone) is shown at the bottom with unique sites marked.

Additionally, a version of B2R incorporating an appropriate restriction site immediately 5' to the initiating ATG was used to create full-length constructs in the pcDNA3 expression vector (Invitrogen) which contain a Kozak sequence followed by an N-terminal epitope tag (FLAG or HA). Transient transfection of COS7 cells with either FLAG- or HA-tagged B2R produces a large protein consistent with a predicted size of 384 kD protein which migrates above a similarly tagged BRCA1 (Figure 4). Thus, we have created full-length, epitope-tagged BRCA2 constructs which can be transiently expressed and detected on Western blot.

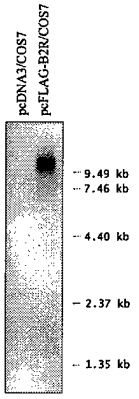

Figure 3: Constructs (pcFLAG-B2R or a control) were transiently transfected into COS7 cells, and RNA was harvested. The Northern blot was probed with a fragment of *BRCA2* (N.B. longer exposures detect endogenous *BRCA2* in control-transfected cells)

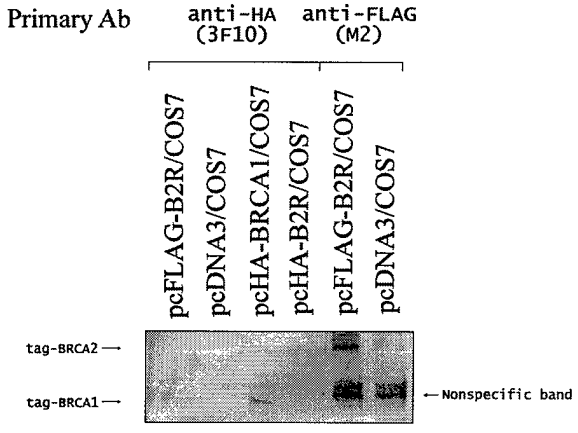

Figure 4: Constructs (FLAG- or HA-tagged BRCA1 or BRCA2) were transiently transfected into COS7 cells, and protein was harvested. A Western blot of these lysates was probed with either anti-HA (3F10, Boehringer Mannheim) or anti-FLAG (M2, Kodak) antibodies. Note the presence of a nonspecific band detected by the anti-mouse secondary antibody used to visualize M2.

Task 2: Generate/test antibodies which detect mouse and human BRCA2 (months 13-24)

A rabbit polyclonal antisera raised against the carboxy terminus of human BRCA2 is now commercially available from Oncogene Research (BRCA2 Ab-2). In order to confirm the identity of the band representing full-length BRCA2, we compared the migration of our full-length FLAG-tagged clone with the prominant band detected by Ab-2 in lysates of the human mammary epithelial cell line MCF7 (data not shown) and the simian SV40-large-T-transformed line COS7 (Figure 5).

Although a commercially available antiserum exists for the detection of human BRCA2 (BRCA2 Ab-2), no reagents have been described which detect murine Brca2. Our lab therefore generated a GST fusion protein containing an amino-terminal fragment of mBrca2 and had purified protein injected into rabbits. The resulting rabbit serum, hereafter designated Ab945, was affinity-purified by covalently binding the original fragment (against which it was raised) to Sepharose beads and performing column purification. Ab945 detects a prominant band in lysates from murine mammary epithelial cell lines NMuMG and HC11, and this band comigrates with the band detected by Ab-2 in COS7 cells (Figure 6). Thus, our evidence suggests that Ab945 is able to detect bona-fide murine Brca2. We further demonstrated that this antiserum is suitable for immunoprecipitation (Figure 7), thus establishing that this reagent has the properties required for crucial aspects of our further experimental design (see below). We thus conclude that we have successfully characterized reagents which allow us to detect either human or murine Brca2.

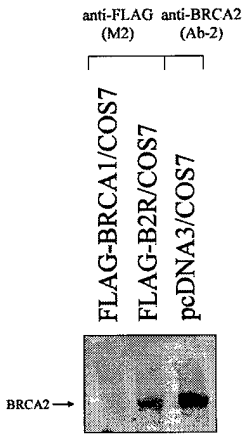

Figure 5: Epitope-tagged BRCA2 comigrates with endogenous BRCA2

Constructs (FLAG-tagged BRCA1 or BRCA2, control vector) were transiently transfected into COS7 cells, and protein was harvested. Protein was separated on an SDS-PAGE gel and blotted onto nitrocellulose. The resulting Western blots were probed with either an anti-FLAG monoclonal antibody or anti-BRCA2 polyclonal antiserum.

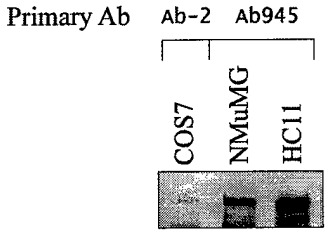

Figure 6: Ab945 detects an band which comigrates with simian BRCA2

Lysate from simian (COS7) or murine (NmuMG, HC11) cell lines were separated by SDS-PAGE and blotted onto nitrocellulose. The resulting Western blots were probed with either anti-human BRCA2 (Ab-2, Oncogene research) or anti-mouse Brca2 (Ab945, see text) antiserum.

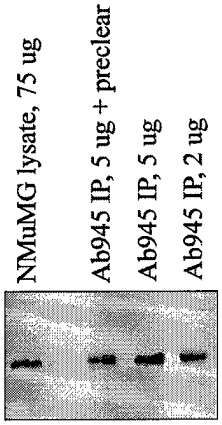

Figure 7: Ab945 Immunoprecipitates mBrca2

Ab945 (2 or 5 μg, with or without preclearing with Protein-A Sepharose) was used to immunoprecipitate from 1 mg NmuMG lysate, and ~2/3 of the resulting material was loaded on an SDS-PAGE gel. A Western blot probed with Ab945 demonstrates that Ab945 can immunoprecipitate a protein which comigrates with the mBrca2 band which is detected in NmuMG lysate.

Task 3: Determine the subcellular localization of Brca2 (months 19-24)

If Brca2 is genuinely involved in the response to DNA damage, it is reasonable to hypothesize that it should be localized to the nucleus. To test this hypothesis, we used affinity-purified Ab945 to examine the subcellular localization of mBrca2 in the murine mammary epithelial cell line NMuMG. Briefly, cells were plated on slides several days before analysis and then fixed in 3% paraformaldehyde/2% sucrose (protocol from Ralph Scully, personal communication). Following permeabilization with Triton X-100, cells were incubated with Ab945 (2 μg/mL in 3% BSA/1X PBS) @37°C x 20'. Following several additional PBS washes, FITC-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch) were added to enable visualization. Confocal fluorescence microscopy demonstrates that Ab945 stains the nucleus of NMuMG cells in a nonuniform pattern with prominant exclusion of the nucleoli (Figure 8). Further, it should be noted that this staining is variable from cell to cell, reflecting significant differences in the localization and/or absolute levels of mBrca2; this may reflect regulation through cell cycle progression (see below) or responsiveness to some other, as yet undefined, signal. We conclude from these experiments that mBrca2 is indeed localized to the NMuMG nucleus, as we hypothesized.

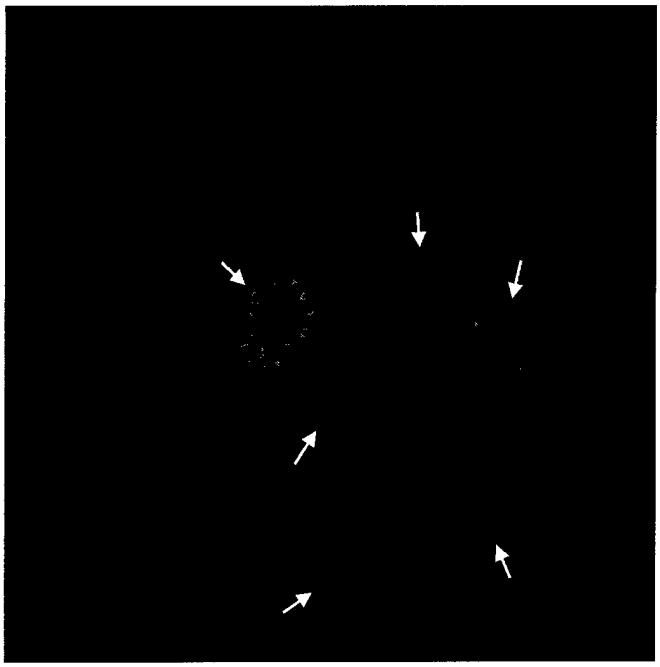

Figure 8: mBrca2 is a nuclear protein which is nonuniformly distributed within the nucleus and which shows significant variation in absolute levels from cell to cell.

NmuMG (a mouse mammary epithelial cell line) cells were plated on slides, fixed, and stained with our anti-mBrca2 antiserum (Ab945). Cells were further stained with a FITC-conjugated anti-rabbit secondary antibody and visualized by confocal microscopy. White arrows indicate the positions of nuclei.

Task 4: Determine whether Brca2 is a phosphoprotein (months 19-24)

Published literature (Scully *et al,* 1997b) suggested a relationship between BRCA1 phosphorylation and the response to DNA damage. We therefore reasoned that determining whether Brca2 is a phosphoprotein would be an important precursor to determining if this posttranslational modification might play a regulatory role in the response to DNA damage. We first incubated one NMuMG plate with ^{32}P-labelled orthophosphate (5 mCi/100 mm plate) for 2 hours prior to lysis in EBC. Following preclearing of the lysate with Protein A-Sepharose, immunoprecipitations were performed with either anti-mBrca2 (Ab945) or control (anti-JAK3, Santa Cruz) rabbit antiserum. In order to identify the expected location of radiolabelled mBrca2, unlabelled NMuMG lysate was loaded onto the same gel. The portion of the gel containing unlabelled lysate was subsequently blotted onto nitrocellulose using semi-dry transfer and probed with Ab945. The results (Figure 9) demonstrate the presence of a phosphoprotein which appears to comigrate with mBrca2 and which is immunoprecipitated by Ab945, but not control, antiserum. We believe that this provides evidence that mBrca2 is in fact a phosphoprotein.

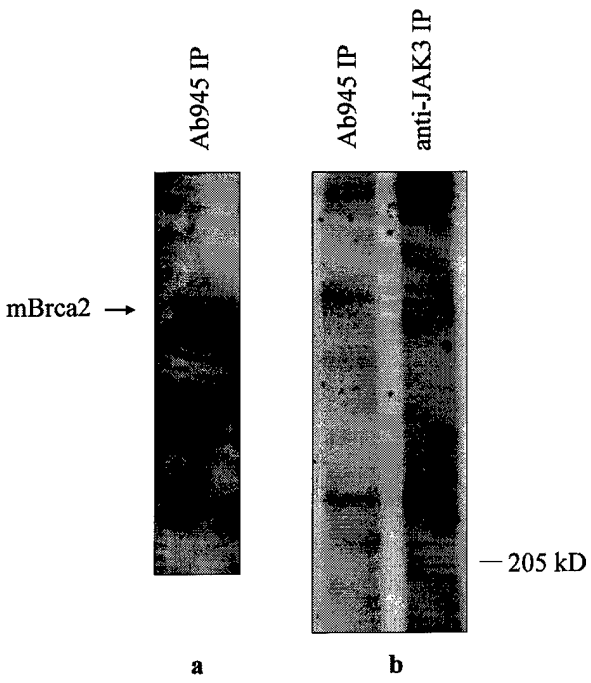

Figure 9: mBrca2 is a phosphoprotein

NmuMG cells were labelled with ^{32}P orthophosphate and lysed. Immunoprecipitations were performed with anti-mBrca2 (Ab945) or control (anti-JAK3) antiserum, and the resulting gel was visualized by autoradiography (figure 9b). To determine the expected migration of mBrca2, a portion of the gel was removed immediately after running and blotted onto nitrocellulose. This Western blot was then probed with Ab945 (figure 9a) and aligned with the autoradiograph.

Task 5: Determine whether BRCA1 interacts with BRCA2 (months 25-36)

We are currently pursuing this question, and it should be noted that the recent first report of an interaction between human BRCA1 and BRCA2 (Chen *et al*, 1998) does not affect our ability to pursue questions related to the murine protein or our ability to use the modular nature of the B2R clone to address the portion of BRCA2 which mediates this interaction.

Task 6: Determine the effect of altered BRCA2 levels on the ability to repair DNA damage. (months 25-36)

Subtask 6.1 Create stable clones which inducibly upregulate BRCA2 expression (months 25-30)

We are currently pursuing the creation of tetracycline-inducible full-length human BRCA2 clones (wild type, 999del5, 6174delT). These clones will be transfected into MCF7 cells containing either the tetracycline transactivator (tTA, cells obtained from

Clontech) or the reverse tetracycline transactivator (rtTA, MCF7 clone transfected with CMV-rtTA construct—data not shown).

Subtask 6.2 Create stable clones which inducibly downregulate Brca2 expression (months 25-30)

Similarly, it is important that we have the ability to downregulate Brca2 levels. To this end, we are continuing to screen HC11 clones which inducibly express antisense Brca2 RNA in the presence of doxocycline (Figure 10). As these clones have not shown the desired degree of downregulation, however (although see Figure 10, clone AS6-5-97 for possible modest regulation), we have designed ribozymes which recognize human and mouse BRCA2 sequence, and we intend to create tet-inducible, stable clones based on these catalytic RNA sequences (see, e.g Hua *et al*, 1996).

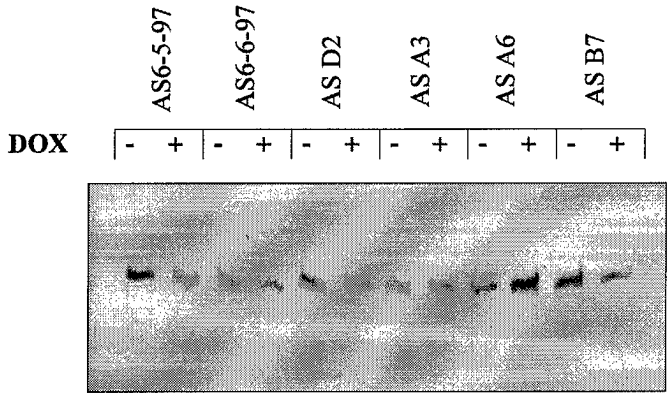

Figure 10: Screening of inducible antisense *Brca2* clones

An HC11 clone which inducibly expressed the reverse tetracycline transactivator (rtTA) was transfected with constructs which contain a segment of antisense *mBrca2* (spanning the translational initiation site) downstream of the tetracycline operator. Stable clones were induced with doxocycline, and Brca2 levels were assayed by Western blot probed with Ab945.

Subtask 6.3 Determine the effect of up- or down-regulating Brca2 on the ability of the cell to respond to DNA damage (months 31-36).

It is anticipated that this analysis will consist of assays for cell survival following gamma- and UV-irradiation, as well as assays for double-stranded break repair (comet assay) and nucleotide excision repair (unscheduled DNA synthesis assay).

Specific Aim II: Determine the role of BRCA2 in Cell Cycle progression

Task 1: Generate a full-length clone of BRCA2 (months 13-18)

See above, under Specific Aim I.

Task 2: Generate/test antibodies for detecting BRCA2 (months 13-24)

See above, under Specific Aim I.

Task 3: Determine whether Brca2 levels are regulated as a function of cell cycle progression (months 19-24).

Our lab has previously demonstrated that *mBrca2* mRNA levels are regulated as a function of cell cycle progression (Rajan *et al*, 1996), and thus we hypothesized that protein levels would be similarly regulated. Although a single report has appeared in the literature during the past year indicating that human BRCA2 protein behaves in this way, to date there has been no report describing the regulation of murine Brca2 protein levels. We therefore serum-starved the murine mammary epithelial cell lines NMuMG and HC11 for >47 hours prior to refeeding in high-serum (20%) growth medium and harvested at various times post-refeed. mBRCA2 protein levels were assayed by probing a Western blot probed with Ab945, and the results are shown in figure 11. These data demonstrate that the murine protein is regulated in a cell-cycle-dependent fashion, thus confirming our hypothesis. In view of the recent report that >60% of cell-cycle-regulated genes in *S. cerevisiae* have identified roles in cell cycle progression (Cho *et al*, 1998), we believe that this evidence suggests the possibility of a direct (as opposed to incidental) role for Brca2 in cell cycle progression .

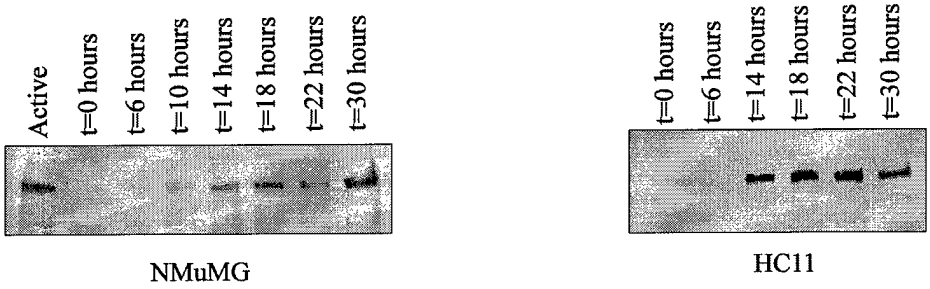

Figure 11: Brca2 is cell-cycle regulated in murine mammary epithelial cell lines

Cells were serum-starved to syncronize them in G0 prior to refeeding. Upon refeeding with 20% serum, cells were harvested at the indicated time points and lysates were analyzed on Western blots (probed with anti-mBrca2 Ab945).

Task 4: Determine the effect of altered BRCA2 levels on cell cycle progression (months 25-36)

Subtask 4.1 Create stable clones which inducibly upregulate BRCA2 expression (months 25-30)

See above, under Specific Aim I.

Subtask 4.2 Create stable clones which inducibly downregulate Brca2 expression (months 25-30)

See above, under Specific Aim I.

Subtask 6.3 Determine the effect of up- or down-regulating Brca2 on cell-cycle progression (months 31-36).

Once subtasks 4.1 and/or 4.2 have been accomplished, cell lines which can be induced to alter Brca2 levels will be analyzed by flow cytometry (i.e. BrdU incorporation, propidium iodide staining for DNA content) to determine whether cells progress normally through the cell cycle. This will directly test our hypothesis that Brca2 levels can affect cell cycle progression.

Training

In addition to the benchwork reflected in this annual report, other training aspects of activity during the last year should be highlighted. First, interaction of the principal investigator (SRM) with his advisor (LAC) and other members of the Chodosh lab is facilitated by a scheduled weekly meeting with the advisor, by biweekly meetings of the BRCA1/BRCA2 working group in the lab (which consists of three graduate students and a postdoctoral fellow), and by weekly lab meetings at which members alternate presenting data and strategy for critical analysis by other members of the lab . In addition, the PI has presented at the Institute for Human Gene Therapy graduate student/postdoc seminar series and in the Cell and Molecular Biology graduate group chalk talk series. A wide variety of relevant seminars are available at the University of Pennsylvania, and several on-campus lectures were delivered by prominent researchers within the BRCA2 field within the period covered by this annual report.

Conclusions

This year represented a critical phase of this training grant, in that the focus of the project shifted to BRCA2 following work on the initial proposal (again, see last annual report for details). We believe, however, that we have demonstrated a successful transition to this different focus and that we are in a strong position to complete the revised statement of work which is reflected in this report. In summary, we have characterized reagents which allow us to detect both mouse and human BRCA2 protein, and we have further demonstrated an ability to immunoprecipitate the murine protein. Using these reagents, we demonstrated that mBrca2 is a nuclear phosphoprotein which is regulated in a cell-cycle dependent manner. Further, we have constructed a full-length human BRCA2 clone which incorporates silent mutations creating unique restriction sites, and we have demonstrated that we can express and detect full-length epitope-tagged versions of this clone. We have reported on recent progress and future plans for creating stable clones which can inducibly alter Brca2 levels. We anticipate that the final year of this grant will result in the successful creation of such clones and their characterization with respect to DNA damage response and cell cycle progression.

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